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Published in:
Journal of Cereal Science

DOI:
[10.1016/j.jcs.2019.102797](https://doi.org/10.1016/j.jcs.2019.102797)

Print publication: 01/09/2019

Document Version
Peer reviewed version

[Link to publication](#)

Citation for pulished version (APA):

Hoyle, AH., Brennan, M., Jackson, G., & Hoad, SP. (2019). Increased grain density of spring barley (*Hordeum vulgare* L.) is associated with an increase in grain nitrogen. *Journal of Cereal Science*, 89, [102797].
<https://doi.org/10.1016/j.jcs.2019.102797>

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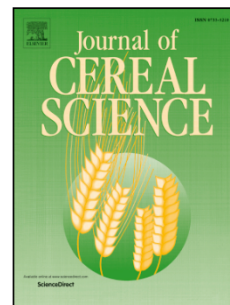
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Accepted Manuscript

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PII: S0733-5210(19)30398-4

DOI: <https://doi.org/10.1016/j.jcs.2019.102797>

Article Number: 102797

Reference: YJCRS 102797

To appear in: *Journal of Cereal Science*

Received Date: 17 May 2019

Revised Date: 27 June 2019

Accepted Date: 28 June 2019

Please cite this article as: Hoyle, A., Brennan, M., Jackson, G.E., Hoad, S., Increased grain density of spring barley (*Hordeum vulgare* L.) is associated with an increase in grain nitrogen, *Journal of Cereal Science* (2019), doi: <https://doi.org/10.1016/j.jcs.2019.102797>.

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Increased Grain Density of Spring Barley (*Hordeum vulgare* L.) is Associated with an Increase in Grain Nitrogen

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Abstract

Barley for malting is evaluated by different grain quality measures, one of these measures is specific weight. An increased specific weight is assumed to lead to higher malt output, however this has not yet been proven. Specific weight is a measure of bulk density, a combination of both individual grain density and the packing efficiency of the grain. Links between specific weight or its components to aspects which may affect malt output have not been investigated. Here we examined correlations between barley grain density and nitrogen content, carbon content, starch content, amylose/amylopectin ratio and starch granule metrics. We show that nitrogen content and the proportional volume, number and surface area of starch B-granules positively correlated with grain density. An equation was built to predict grain density from grain nitrogen and the proportional volume of starch B-granules; this described 47% of observed variation in grain density. An independent validation of the equation indicated that nitrogen content alone was sufficient to successfully estimate grain density. As nitrogen content is consistently positively correlated with grain density and hence specific weight, a high specific weight could be unfavourable for some malting end-uses which require low grain nitrogen. Achievement of high specific weight must therefore carefully consider end-user requirements.

Keywords: Barley (*Hordeum vulgare* L.); specific weight; grain density; grain composition

1. Introduction

Barley (*Hordeum vulgare* L.) is the main cereal used in the malting process, whereby grain undergoes steeping, germination and kilning to produce malt (Gupta et al., 2010). Steeping increases the moisture content of grains from their storage conditions of typically 12% to greater than 40%, triggering germination and a cascade of physical and biochemical modifications within the endosperm (Briggs, 1998). These modifications include the accumulation of malt enzymes, cell wall degradation and physical changes such as softening of the grain (Briggs, 1998). Kilning arrests germination by drying the malt at elevated temperatures, stabilising the enzymes produced which are harnessed in downstream processes. In the UK, malt is primarily used in the brewing and whisky distilling industries, but it is also used in some food products and is an important export for the UK (Baik and Ullrich, 2008). Barley grain is graded on numerous quality criteria prior to acceptance for malt production. The strict criteria that have to be met by growers supplying for the malting industry result in a higher price for high quality barley. One of these grain quality criteria is specific weight (SW); one of the longest standing measures of grain quality. It is a measure of bulk density, that is, the weight of grain per unit volume (Briggs, 1998). A high SW is thought to be associated with higher malting efficiency and is therefore a breeding target. In a previous study it was shown that the SW of barley grains is a product of two components: single grain density (SGD) and packing efficiency (PE) (Hoyle et al., 2019).

It is important to distinguish between bulk density, SGD and grain hardness because they are distinct measures (Psota et al., 2007). Bulk density describes the mass of grain in a given volume, whereas SGD describes the density of an individual barley grain. Grain hardness is harder to define in barley, however in wheat it is associated with milling energy. Hardness is not a measure or indicator of SGD, however in wheat it has been shown that soft and hard wheat cultivars have a large overlap in SGD

Links between SGD and barley grain composition have not previously been studied. The endosperm is the largest grain tissue comprised of two components, the aleurone and the starchy endosperm (Evers et al., 1999). The starchy endosperm forms the majority of this tissue, in which endosperm cells store nutrients which are mobilised upon the onset of germination to sustain the embryonic axis (Evers et al., 1999). Cell walls in the barley endosperm are abundant in mixed linkage β -glucans (Evers and Millar, 2002). The major constituents of barley grains are starch (60-80%), nitrogenous compounds (9-13%), lipids (1-2%) and water (10-15%) (Asare et al., 2011). Starch is composed of two different types of D-glucose polysaccharides; amylose and amylopectin (Jeon et al., 2010). Amylose is a linear polymer of 1,4-linked α -glucose residues with minor branching, whereas amylopectin is a highly branched polymer consisting of 1,6-linked α -glucose residues (Jeon et al., 2010). These two polysaccharides are stored in the form of semi-crystalline starch granules in the endosperm, in either A- or B-type granules. These granules differ in their size, shape and composition. The larger, biconvex A granules have a diameter of between 8 and 30 μm , whereas the smaller, spherical B granules have a diameter of less than 8 μm (Evers et al., 1999). The size distribution of barley starch granules exhibits a bimodal distribution distinguishing between the two granule types. The majority of nitrogenous compounds in barley grains are proteins, with hordeins being the most prevalent protein (Gupta et al., 2010).

Relationships between both grain physical characteristics, grain composition and malt quality parameters have long been studied (Agu et al., 2007). Physical characteristics that affect malt quality include grain size and size uniformity, weathering, and skinning (Fox, 2010). Compositional attributes such as starch content and composition are of high importance for determining malt quality, with the ratio of amylose and amylopectin affecting starch gelatinisation properties (Fox, 2010). The gelatinisation of starch is important for malt quality because the rate of starch hydrolysis by malt enzymes post-malting is increased once starch granules become soluble through gelatinisation

to be reached in order to ensure complete gelatinisation (Macgregor et al., 2002). Protein content is also important in regard to malt quality and is influenced by both growing conditions and genotype (Fox, 2010). A high protein content is considered detrimental for malting efficiency as it can reduce the proportion of starch in the endosperm. However, there must be sufficient amino acids present to sustain yeast, particularly for brewing (Fox, 2010).

It is important to characterise any correlations between SGD and grain composition, in order to determine whether increasing SGD and hence SW can either confer potential benefits, or detract from malting efficiency. If an increased SGD correlates with compositional characteristics thought to improve malt output, such as increased starch content or low protein content, this would provide evidence that increased SW truly is a good indicator that grain is of malting quality. Conversely, if an increased SGD correlates with traits that compromise malt output, such as excessive protein content or a higher ratio of B starch granules, it could indicate that SW is unlikely to indicate whether grain is of high malting quality.

In this study the aims were (1) to examine correlations between quantitative changes in grain composition and SGD, (2) to build an equation to predict SGD from grain composition to understand the contributions of compositional aspects to SGD and (3) to test the accuracy and efficacy of the equation using an independent validation grain sample.

2. Materials and Methods

2.1 Materials

Barley grains of five malting cultivars (Sienna, Laureate, Concerto, Olympus and Odyssey) from the Agriculture and Horticulture Development Board's (AHDB's) Recommended List (RL) 2016/17 were used in this study. These cultivars were selected due to their phenotypic range in grain size. SW and

using a 2.50 mm slotted sieve, with 19.05 mm long slots and shaken for 20 s. Barley grains from a separate sample of Sienna were used to validate the equation derived from the original five cultivars. This sample was a commercial bulk provided by Bairds Malt and grown during the 2017 season, which contains spring barley grown across Scotland.

2.2 Sampling

In order to obtain a representative sample of grains to analyse, 350 g grain samples were sequentially sieved by hand into a range of size fractions using a stack of slotted 3.25, 3.00 and 2.75 mm sieves, with 19.05 mm long slots. The weight of grain in each size fraction designated; large (> 3.25 mm), medium (3.25 to 3.00 mm), small (3.00 to 2.75 mm) and very small (< 2.75 mm) was recorded using a Kern analytical balance PLJ 3500-2NM (accuracy ± 0.01 g). Three 100-grain samples were weighed from each size fraction, and the mean grain weight used to estimate the total number of grains in each fraction. A number of grains proportional to the total number of grains from each fraction were chosen at random, to give 300-grain samples which were representative of the total larger bulk sample, for each cultivar used in this study.

2.3 Grain density and sample stratification

On each 300-grain sample, grains were individually weighed using a Mettler AE 160 electronic balance (Mettler-Toledo, accuracy ± 0.0001 g). The volume of individual grains was measured by placing them in a submersed, but suspended crucible in a beaker of water. The change in weight on the balance due to the buoyant force acting on the grain is equal to the weight of water displaced and hence the volume of the grain (Archimedes' principle). To create five density classes within each cultivar, grains were ordered by density. Density classes were created by grouping the 60 least dense grains and so on until the 60 most dense were left, creating 25 samples in total (Fig. 1A). In order to visualise the endosperm and in particular the starch granules within endosperms of different

2.4 Elemental and starch analyses

Twenty grains from each 60-grain sample were milled into a fine powder using a ball mill (Mixer Mill MM 200, Retsch, Germany) for compositional analyses. The proportion of carbon and nitrogen in the grain, typically referred to as carbon and nitrogen contents, were determined with a FLASH 2000 Organic Elemental Analyzer (Thermo Scientific). Total starch content and the ratio of amylose to amylopectin were measured using Megazyme kits: Total Starch Assay Kit (K-TSTA-100A) and Amylose/Amylopectin Assay Kit (K-AMYL) (Megazyme Ltd. Ireland) using the assay procedures provided by the manufacturer. Starch analyses are reported as a percentage of starch content for amylose and amylopectin (w/w) and 'as is' basis (g/100g) for total starch content.

2.6 Starch granule isolation and size distribution analysis

Starch was purified separately from three 10-grain subsamples of the 60-grain samples according to the "method 1" in Verhoeven et al. (2004) and then freeze-dried using an Alpha 1-4 LSCplus (Christ, Germany) at -20°C in a vacuum overnight prior to analysis. A known mass of purified starch was dispersed in 100 ml of Isoton II Diluent (Beckman Coulter, United States). The size distribution of starch granules was determined with a Multisizer 4e Coulter Counter (Beckman Coulter) with a 70 µm aperture tube. The Multisizer measures the volume of each starch granule passing through its aperture between two electrodes using the Coulter Principle. In excess of 200,000 particles were measured per sample, and size frequency distributions were recorded in 400 logarithmically spaced bins between the diameter range of 1.4 µm to 42 µm. The number of granules passing through the aperture was counted and the surface area of these estimated by using the surface area of a sphere with the same measured volume. Therefore results of starch granule analysis include B-granule: number, volume and surface area. These are all reported as a percentage of the total for all measured granules. Consistent with a previous study by Chmelik et al. (2007), we used a threshold of

alternative method for estimating the proportion of A- and B-type granules, based on a mixed distribution curve-fitting method, similar to that described in Tanaka et al. (2017). However, the mixed distribution was not able to accurately fit our size distributions of barley starch granules, as there was very little overlap in the A- and B-type granule distributions.

2.7 Statistical analysis

Data analysis was carried out in R software version 3.4.1 (R Core Team, 2017). Analysis of variance ($\alpha = 0.05$) was used to determine whether grain density class and cultivar had a significant effect on SGD, elemental analyses and starch analyses. Where a significant effect was indicated, a post-hoc Tukey's Honestly Significant Difference (HSD) ($\alpha = 0.05$) test was conducted to determine which samples differed from one another. This is indicated by different letters in the results table. A stepwise linear regression was performed in R using the 'olsrr' package to determine which variables significantly contributed to predicting SGD and therefore should be included in the equation (Hebbali, 2018). The response variable was SGD, and the dependent variables were: nitrogen, carbon, total starch, amylose, and B granule volume. Independent variables were selected based on p-value, the threshold for a variable to enter the equation was $P < 0.1$ and to exclude a variable from the equation was $P > 0.3$ in accordance with the default setting on the 'olsrr' package (Hebbali, 2018). The correlation between measured grain density and calculated grain density was determined using Pearson's product-moment in the R package "corrplot" (Wei and Simko, 2016).

3. Results

Single grain density and compositional variables including: nitrogen (N) content, carbon (C) content, total starch content, amylose/amylopectin ratio and starch B granule; number, volume and surface area were measured on the 25 samples created by stratifying 300 grains from each cultivar into five

3.1 Effect of single grain density on grain composition

Table 1 summarises the means and standard deviations of SGDs and compositional aspects of the five different density classes: very low, low, medium, high and very high. Stratifying samples by density created a range of 1.16 g cm^{-3} to 1.27 g cm^{-3} . No differences in C content were observed between the different density classes; this measure only had a small range of 39.85% to 40.23% from the medium and low density classes. Density had a significant effect on grain N content, with N content sequentially increasing with each density class. Nitrogen content of the very low and low class was 1.36% and 1.40%, respectively. These were both significantly ($P < 0.05$) lower than that of the very high class (1.53%). Starch content did not differ significantly among density classes. All starch contents were within the range from 58.62 g/100 g to 58.78 g/100 g. Amylose content was highest in the very low density class (20.76%) which was significantly greater ($P < 0.05$) than the high density class (16.98%). The inverse was the case for amylopectin content. No significant differences were observed in the three measures of B granule content, however the values increased sequentially from the very low density class to the very high density class as follows: B granule number 97.21% to 97.56%, B granule volume 20.20% to 23.55% and B granule surface area 54.79% to 59.05%.

3.2 Effect of cultivar on grain composition

Table 2 summarises the means and standard deviations of SGDs and compositional variables of the five spring barley cultivars; Sienna, Laureate, Concerto, Olympus and Odyssey. Mean SGD ranged from 1.24 g cm^{-3} for Sienna to 1.19 g cm^{-3} for Concerto, although no significant differences were observed among cultivars. No significant differences were observed in grain C or N contents among cultivars. Odyssey had both the lowest C and N contents at 39.85% and 1.41%, respectively. Sienna had the highest C content (40.22%), and Laureate the highest N content (1.50%). The total starch

0.05). The ratio between amylose and amylopectin did not differ significantly among the cultivars measured. The three measures of starch B granules; number, volume and surface area shown as a percentage of total granules, all showed similar patterns across the cultivars. Starch B granule number was highest in Laureate (97.75%) which was significantly higher ($P < 0.05$) than Odyssey (97.28%). Concerto's B granule number (96.75%) was significantly lower ($P < 0.05$) than the other four cultivars. Concerto had significantly lower B granule volume and surface area (17.86% and 51.15%, respectively) ($P < 0.05$) than the other four cultivars. Laureate had the highest B granule volume (24.27%) and surface area (60.51%), but this was only significantly higher than Concerto ($P < 0.05$).

3.3 Correlations between compositional traits

The significance of correlations between SGD and different compositional variables were analysed and a matrix of the Pearson correlation coefficients (r) are given in Table 3. Corresponding p-values are in Table S2.

The highly significant positive correlation between SGD and N content ($r = 0.61$, $P < 0.01$, Fig 2A.) highlights the effect of SGD on N content which was observed in 3.2. In addition to this there are significant correlations between SGD and B granule volume ($r = 0.55$, $P < 0.01$, Fig 2D.), B granule number ($r = 0.51$, $P < 0.01$) and B granule surface area ($r = 0.55$, $P < 0.01$). These are the only two variables with which SGD is significantly correlated. Single grain density did not correlate with either C content or starch content (Fig 2B, Fig 2C). Alongside correlating with SGD, B granule volume positively correlated with N content ($r = 0.44$, $P < 0.05$), starch content ($r = 0.43$, $P < 0.05$) and was negatively correlated with amylose content ($r = -0.57$, $P < 0.01$). B granule surface area also negatively correlated with amylose content ($r = -0.52$, $P < 0.01$).

3.4 Predicting single grain density from compositional traits

Independent variables which were calculated from one another (amylose/amylopectin) and those which displayed high levels of collinearity (B granule; volume, number and surface area) are represented only once by amylose and B granule volume, respectively. Stepwise regression analysis removed all independent variables apart from N content (%) and B granule volume (%). The independent variables removed were C content (%), amylose (%) and total starch (g/100 g). The predictive equation derived from this analysis was:

$$\text{Density (g cm}^{-3}\text{)} = 0.779 + 0.224 \cdot \text{N} + 0.005 \cdot \text{B}$$

N - Nitrogen Content (%)

B - Starch B granule volume (%)

Nitrogen content alone described 37.1% of the variation in SGD. The addition of B granule volume to the equation resulted in the r^2 value increasing from 0.371 to 0.473, with the final equation describing 47.3% of the variation in SGD. The relationship between measured grain density and the predicted grain density using this predictive equation on the original 25 samples was highly significant ($r^2 = 0.473$, $P < 0.001$, Fig. 3a). Each cultivar is likely to have a slightly different slope as demonstrated in Fig S1, therefore this predictive equation may need to be altered for highly accurate predictions to account for different genotypes.

3.5 Validation of the density equation

A separate sample of commercial barley grains from the cultivar Sienna was stratified in the same way to create five samples of differing densities to provide samples for equation validation. These were analysed for N content and starch B granule volume. The relationship between measured grain density and the predicted grain density (using the predictive model built in 3.4) of the validation sample was not significant ($r = 0.83$, $P = 0.085$). However when a model was built from the original

$P < 0.05$, Fig.3b). When comparing grain density with B granule volume and measured grain density, no significant correlations were observed.

4. Discussion

It is widely known that the composition of barley grains affects malt quality, which has been summarised by Fox (2010). Starch structure affects gelatinisation temperature and consequently hot water extract (Macgregor et al., 2002), high protein content correlates with reduced starch levels but low protein content is detrimental for yeast nutrition. However, how the composition of barley grains affects SGD, a component of SW (Hoyle et al., 2019), has not previously been studied. Linking this fills a gap in the knowledge between grain quality and malting. Furthermore, lessons could be applicable to other cereal species such as oats and wheat which use SW as a measure of grain quality for alternative end-uses. If SGD also positively correlates with nitrogen content in wheat, this could reinforce the importance of SW as a quality measure in bread making, since both the quantity and quality of protein in wheat is important in this process (Johansson et al., 2001).

In this study we stratified grain samples from five spring barley cultivars into five different grain density classes, to create a large range in SGDs across 25 samples. Compositional analyses were performed on these samples to determine how compositional traits vary with SGD and to build a predictive equation to quantitatively link composition to SGD. We demonstrated that N content, which is often used as an estimate of protein content, is strongly correlated with SGD across the 25 samples. This is a novel finding since N or protein content have not previously been linked to the density of barley or other cereal grains. This link between protein content and one of the components of SW is an integral step to understanding how SW may affect malting, brewing and distilling. Generally, protein content is negatively correlated with available carbohydrates which

inadequate starch degradation through interfering with starch degradation enzymes and also enveloping starch granules in the endosperm (Yu et al., 2019). Therefore a high protein content can reduce the amount of fermentable sugars produced during mashing. However, too low a protein content could mean there are too few amino acids formed through proteolysis during mashing for yeast metabolism to occur (Gupta et al., 2010). Furthermore, through the analysis of a validation sample, N content also showed a positive correlation with SGD, demonstrating that this link between N and SGD is consistent for the samples tested.

In addition to the relationship between N content and SGD in the original samples, starch B granule volume also showed a strong positive relationship with SGD. The conversion of starch into fermentable sugars is an integral part of brewing, therefore the rate of starch gelatinisation and hydrolysis are important factors to consider (Gupta et al., 2010). Barley starch A and B granules are different sizes and have an altered composition of polysaccharides, therefore they have distinctive physical and chemical properties (Jaiswal et al., 2014). Starch B granules have a lower proportion of amylose compared to A granules, as confirmed by the significant negative correlation observed between B granule volume and amylose in this study. At lower temperatures (35°C) the smaller starch B granules gelatinise more quickly than the larger A granules, but at higher temperatures more similar to that used in the mashing process (65°C) the opposite occurs (Gupta et al., 2010). Consequently the hydrolysis of starch B granules into soluble sugars during mashing occurs at a slower rate than A granules, which can cause problems in the brewing process (Macgregor and Ballance, 1980). Therefore in these samples an increased SGD is associated with potentially detrimental starch granule characteristics for malting. However when the validation sample was analysed this relationship between B granule volume and SGD was not observed. This demonstrates that this relationship doesn't always hold true across sites. The reason this relationship may not have held true may be due to the different environments this validation sample was grown in, since

B granules (Tester, 1997). The synthesis of A granules starts soon after anthesis and B granule synthesis is initiated later such that throughout grain filling B granule number increases (Lindeboom et al., 2004).

No relationships were observed between SGD and both C content and total starch content of barley grains. It has been reported that starch content negatively correlates with protein content in barley grains. Therefore since N content positively correlates with SGD it might have been expected that total starch content would display the opposite trend however, this was not the case. Other studies have also shown that starch content does not always correlate with protein content (Yu et al., 2019, 2017).

The equation derived to predict SGD from grain composition is the first of its kind and went some way towards accurately predicting SGD through using just nitrogen content and B-granule volume. The aim of this equation was to better understand the basis of barley SGD and therefore this component of SW. To test both the accuracy and efficacy of this equation at predicting density for samples from different origins, the equation was applied to an independent grain sample for validation. The equation generally under-estimated density for samples with a higher measured density, and the gradient of this was not parallel with the original dataset. However, a model was then built from the original dataset using N alone to predict density, and when applied to this validation dataset, improved predictions were made. Different environmental conditions are known to have large effects on starch granule composition, functionality and proportions. The grains used to build the model came from Docking, Norfolk, whereas the validation set came from a bulk of harvests from across Scotland. Within one site the inclusion of the B-granule volume achieved a more precise model specifically for those data. However when including grains from diverse locations, the proportion of B-granule volume have less explanatory power, potentially due to the

internal structure created by these cell walls. The density value used for each density class was the mean of the 60-grain sample, however with compositional analyses used different methods, the full 60 grains couldn't be used for each analysis. Therefore this 60-grain sample had to be subsampled for each method, despite the subsamples all having similar densities this could have potentially introduced some error since each subsample could have had a slightly different composition.

Since the predictive equation described 47% of the observed variations in SGD, additional grain characteristics need to be measured to fully describe SGD. Further studies could move away from composition and examine the internal architecture of the endosperm. These would investigate how the endosperm cell architecture influences density or endosperm porosity. Porosity is known to affect grain density and hardness in wheat, so is therefore likely to influence barley SGD (Dobraszczyk et al., 2002). Grain hardness has previously been shown to have a relationship with malt quality. Therefore this could provide further links between characteristics affecting SW through SGD and ultimately influencing malt quality (Psota et al., 2007). In addition to porosity, the proportion of husk tissues to endosperm may also influence SGD and SW. In oats it has been demonstrated that the density of groat alone is greater than that of the oat kernel, implying the hull negatively contributed to SGD (Doehlert et al., 2009).

Specific weight is one of the longest standing measures of grain quality, and is used across several cereals. In barley, this may be because it is easy to measure, the equipment is cheap and the results are straightforward to interpret, rather than because of its accuracy as a malt quality indicator (Manley et al., 2009). Consequently, the value of SW as a measure of malting grain quality has been disputed and understanding what contributes to this measure is essential in order to determine whether SW could influence malting efficiency or productivity (Hoyle et al., 2019). This study demonstrated links between grain composition and SGD, one of the components of SW. We have

important to determine how this SW has been achieved. If a high SW has been achieved due to an elevated N content and B granule volume this does not necessarily mean this grain is of the highest value for malting and other downstream uses. Therefore a more detailed understanding into how SGD or PE has resulted in a SW value needs to be known to link SW through to malt quality. It is possible that in the future, SW as a measure could be altered or replaced to account for these problems, a potentially improved measure of quality could be starch content and/or quality per volume of grain.

5. Acknowledgements

The authors thank Dr David Seung of the John Innes Centre for assisting with starch granule analysis and allowing us to use his particle size analyser for the work outlined. Without David this important part of the work would not have been possible. The authors also thank the AHDB (project number: 21130047) for both the financial support of this work and the provision of samples. We also acknowledge funding from the Scottish Government's Rural and Environmental Science and Analytical Services (RESAS) division.

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454

ity, elemental analysis and starch analyses on different density

| | Nitrogen (%) | Carbon (%) | Total Starch (g/100g) | Amylose (%) | Amylopectin (%) | B granule number (%) | B granule volume (%) | B granule surface area (%) |
|--------------------|--------------------------|-------------------------|--------------------------|--------------------------|--------------------------|-------------------------|-------------------------|-------------------------------|
| 0022 ^d | 1.36±0.056 ^b | 40.03±0.30 ^a | 58.64±0.72 ^a | 20.76±1.71 ^a | 79.24±1.71 ^b | 97.21±0.47 ^a | 20.20±2.95 ^a | 54.79±4.48 ^a |
| 0019 ^c | 1.40±0.026 ^b | 40.23±0.16 ^a | 58.69±0.16 ^a | 18.57±2.32 ^{ab} | 81.43±2.32 ^{ab} | 97.29±0.57 ^a | 22.02±3.22 ^a | 56.88±4.81 ^a |
| 0018 ^{bc} | 1.46±0.055 ^{ab} | 39.85±0.45 ^a | 58.78±0.59 ^a | 18.34±2.14 ^{ab} | 81.66±2.14 ^{ab} | 97.44±0.36 ^a | 22.40±2.94 ^a | 57.76±3.83 ^a |
| 0016 ^{ab} | 1.47±0.067 ^{ab} | 40.14±0.30 ^a | 58.75±1.39 ^a | 16.98±1.01 ^b | 83.02±1.01 ^a | 97.47±0.48 ^a | 23.09±3.40 ^a | 58.58±4.64 ^a |
| 0017 ^a | 1.53±0.103 ^a | 39.92±0.30 ^a | 58.62±0.92 ^a | 19.40±1.84 ^{ab} | 80.60±1.84 ^{ab} | 97.56±0.26 ^a | 23.55±2.19 ^a | 59.05±2.60 ^a |

on a wet weight basis and are means of five different cultivars ± standard deviation. When comparing mean values within a column those followed are significantly different from one another (p<0.05).

Table 1. Grain density, elemental analysis and starch analyses on five spring barley cultivars^b

| Grain density cm ⁻³ | Nitrogen (%) | Carbon (%) | Total Starch (g/100g) | Amylose (%) | Amylopectin (%) | B granule number (%) | B granule volume (%) | B granule surface area (%) |
|-----------------------------------|-------------------------|-------------------------|--------------------------|-------------------------|-------------------------|----------------------------|----------------------------|----------------------------------|
| 24±0.040 ^a | 1.42±0.067 ^a | 40.22±0.23 ^a | 59.33±1.05 ^a | 19.81±1.43 ^a | 80.19±1.43 ^a | 97.67±0.27 ^{ab} | 23.28±2.30 ^a | 59.37±3.05 ^a |
| 21±0.039 ^a | 1.50±0.085 ^a | 39.88±0.31 ^a | 58.73±0.52 ^{ab} | 18.49±2.19 ^a | 81.51±2.19 ^a | 97.75±0.15 ^a | 24.27±1.75 ^a | 60.51±1.78 ^a |
| 19±0.046 ^a | 1.42±0.038 ^a | 40.09±0.39 ^a | 58.30±0.40 ^{ab} | 20.65±1.43 ^a | 79.35±1.43 ^a | 96.75±0.35 ^c | 17.86±1.96 ^b | 51.15±3.11 ^b |
| 22±0.041 ^a | 1.46±0.144 ^a | 40.13±0.29 ^a | 59.17±0.58 ^a | 17.35±1.97 ^a | 82.65±1.97 ^a | 97.53±0.17 ^{ab} | 24.16±1.97 ^a | 59.44±2.21 ^a |
| 21±0.047 ^a | 1.41±0.051 ^a | 39.85±0.32 ^a | 57.94±0.35 ^b | 17.74±1.74 ^a | 82.26±1.74 ^a | 97.28±0.12 ^b | 21.69±0.90 ^a | 56.59±1.19 ^a |

^a Data are reported on a wet weight basis and are means of five different density grades per cultivar ± standard deviation. When comparing mean values within a cultivar, values followed by different letters are significantly different from one another (p<0.05).

Correlation matrix of Pearson correlation coefficients (r) for grain density, elemental analysis and amylose.

| | Grain density (g cm ⁻³) | Nitrogen content (%) | Carbon content (%) | Starch content (%) | Amylose content (%) | B granule volume (%) | B granule number (%) | B granule surface area (%) |
|-------------------------------------|-------------------------------------|----------------------|--------------------|--------------------|---------------------|----------------------|----------------------|----------------------------|
| Grain density (g cm ⁻³) | 1 | 0.61** | -0.01 | 0.2 | -0.31 | 0.55** | 0.51** | 0.55** |
| Nitrogen content (%) | | 1 | 0.1 | 0.09 | -0.37 | 0.44* | 0.34 | 0.41* |
| Carbon content (%) | | | 1 | 0.19 | 0.05 | -0.17 | -0.19 | -0.18 |
| Starch content (%) | | | | 1 | -0.12 | 0.43* | 0.34 | 0.39 |
| Amylose content (%) | | | | | 1 | -0.57** | 0.37 | -0.52** |
| B granule volume (%) | | | | | | 1 | 0.94*** | 0.99*** |
| B granule number (%) | | | | | | | 1 | 0.98*** |
| B granule surface area (%) | | | | | | | | 1 |

"**" were significant at P < 0.001, P < 0.01 and P < 0.05

Figure Captions

Fig 1. Range of grain densities created by stratifying grain samples **(A)** from five cultivars into five individual classes according to density. Concerto 1 referring to the least dense 60 grains of the 300 grain sample and Concerto 5 referring to the most dense 60 grains. Scanning electron micrographs from cracked endosperms of spring barley cultivar Laureate, **(B)** high density and **(C)** low density. Scale bar = 10 μm . The arrow in fig 1B points to a large starch A-granule and the arrow in fig 1C points to a small starch B-granule.

Fig 2. Regression analysis of grain density against grain constituents; (A) nitrogen content ($r = 0.61$, $P = 0.001$), (B) carbon content ($r = 0.21$, $P = 0.948$), (C) starch content ($r = 0.06$, $P = 0.348$) and (D) B granule volume ($r = 0.53$, $P = 0.004$). ■, Concerto; ●, Laureate; ▲, Odyssey; +, Olympus; ×, Sienna. Shaded areas represent the 95% confidence interval of the regression.

Fig 3. Scatter plots of measured grain density using Archimedes' Principle and predicted grain density using the predictive equation built in part 3.4 for **(A)** the original 25 samples from five cultivars and **(B)** using N alone to predict the density of the validation five samples. The regression line (black) in both parts if formed from the original dataset, with the confidence interval of 95% shown by the grey shaded area.

Fig S1. Scatter plot of measured grain density using Archimedes' Principle and predicted grain density using the equation built in part 3.4 for 25 samples from five cultivars.

Figure 1

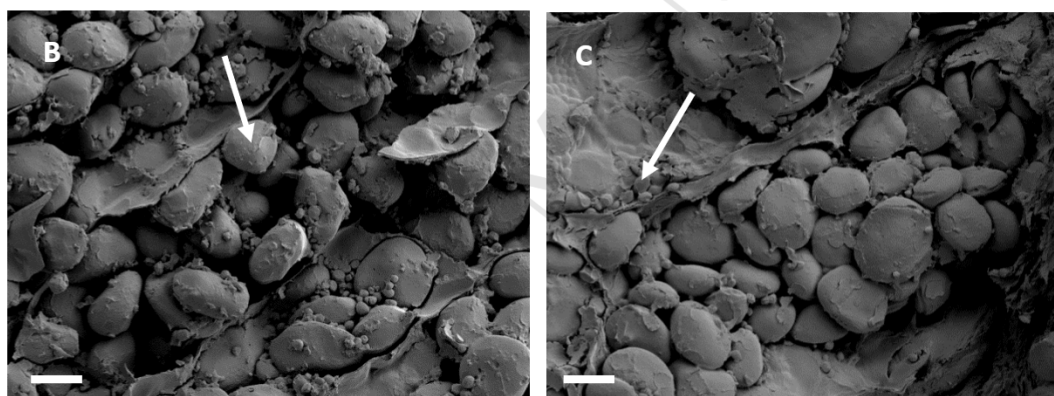
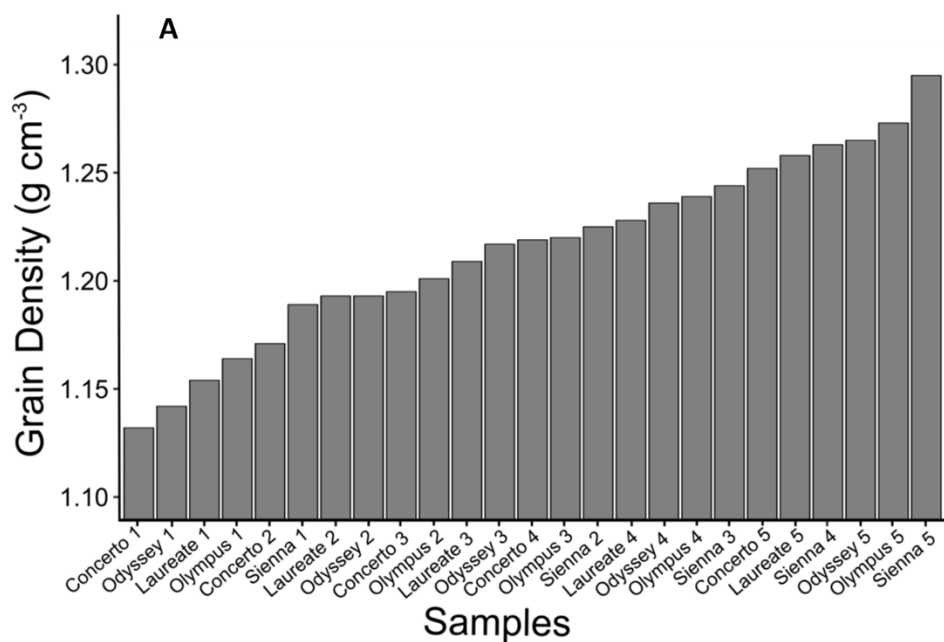


Figure 2

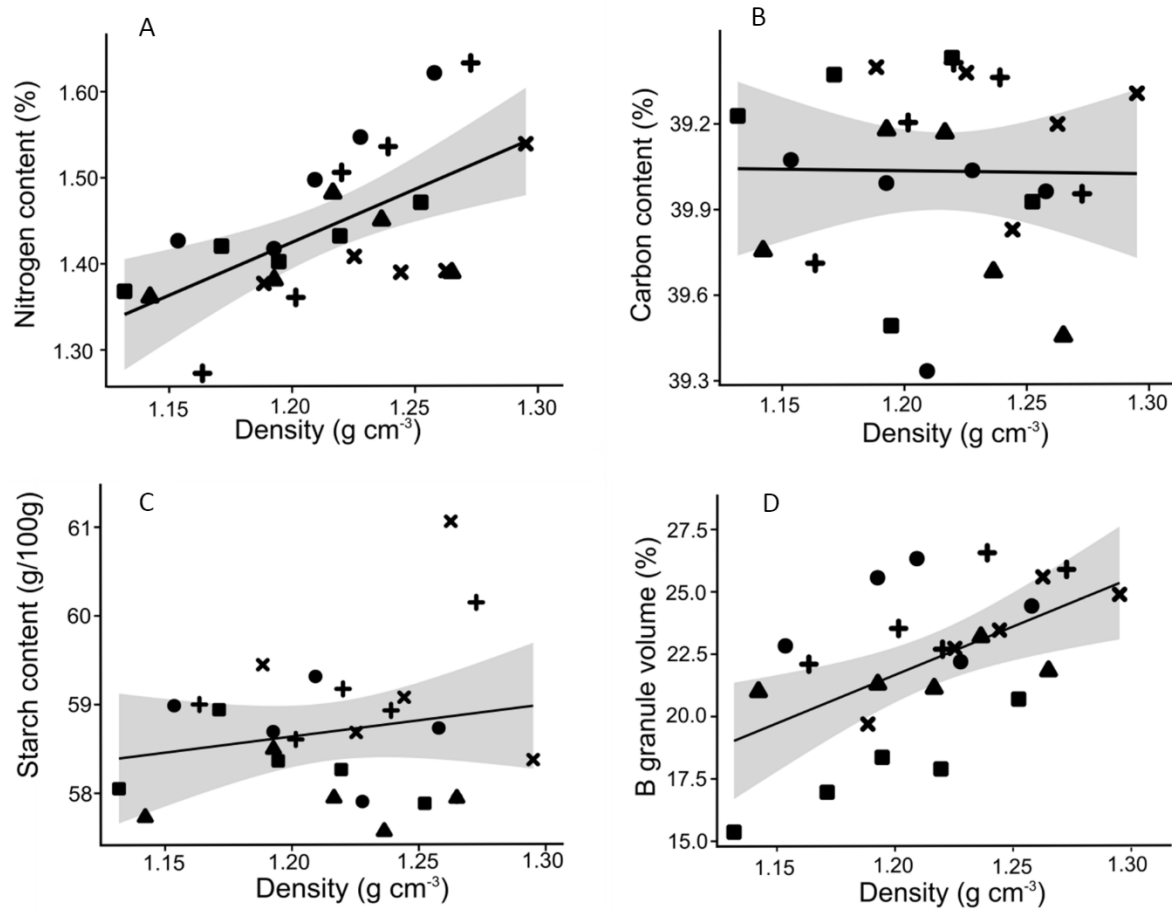
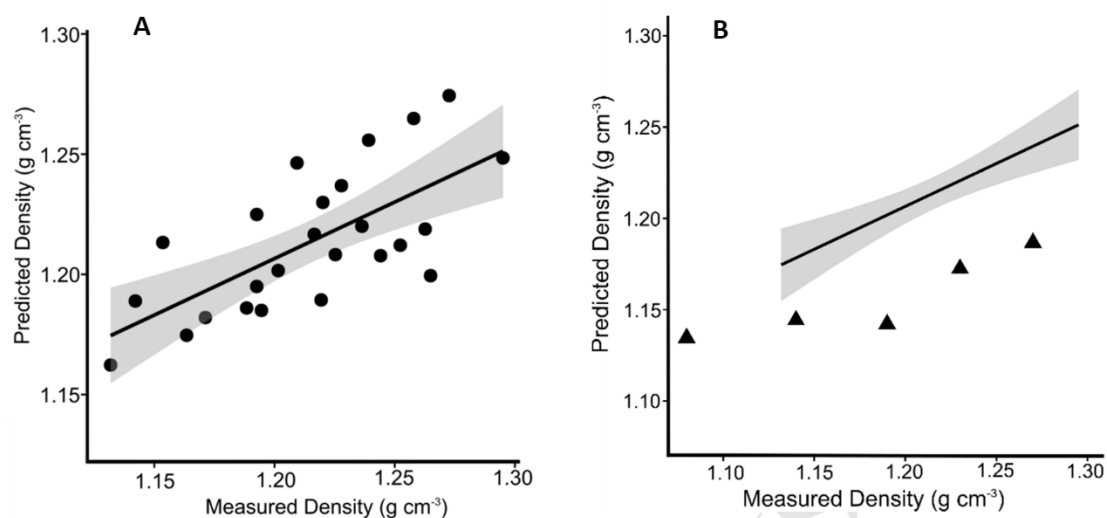


Figure 3

Increased Grain Density of Spring Barley (*Hordeum vulgare* L.) is Associated with an Increase in Grain Nitrogen

Highlights

- Grain composition is linked to single grain density (SGD) in barley grains
- Nitrogen content and starch B-granules positively correlate with SGD
- Nitrogen content explains the largest proportion of variation in SGD
- High specific weight may not be beneficial for all end-users

